QUANTITTIVE DETERMINATION OF AMINO SUGARS IN BACTERIAL LIPOPOLYSACCHARIDES

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A modification of the method of quantitatively determining amino sugars in polysaccharide preparations of bacterial origin is described which excludes the influence of the mixture of amino acids and monosaccharides on the result of the analysis. The modification is based on differences in the extraction capacity of organic solvents in relation to the various chromophores obtained in the Elson-Morgan reaction from amino sugars and from the mixture of amino acids and monosaccharides. It has been found that the modification described is distinguished by a high sensitivity, specificity, and reproducibility of the results. The standard deviation calculation for 10 determinations does not exceed 4%. The method is simple to perform and it requires uncomplicated equipment and small amounts of reagents.

In view of the exceptionally important role that amino sugars play in the structure of biopolymers and in biochemical processes, reliable methods for the quantitative determination of these monosaccharides in materials under investigation are necessary. The most sensitive method and that most frequently used for the analysis of micro amounts of free amino sugars in biopolymers is the Elson–Morgan method [1]. At the present time, several modifications of it have been developed [2-4] that are directed mainly to standardizing the conditions of the analysis, but the main disadvantage of the method and of its modifications is the nonspecificity of the reaction for amino sugars when they are present simultaneously in a mixture with other monosaccharides and with amino acids.

In the present paper a new modification of the method, directed to the solution of this problem is proposed. With this aim, we have determined the degree of influence of monosaccharides and amino acids on the results of the analysis of the amino sugars. Free amino sugars form a colored solution with a maximum absorption at 530 nm (Fig. 1).

It has been shown that neither monosaccharides nor amino acids give a positive Elson–Morgan reaction, but their mixtures (minimum concentration of each component 15 μg) form a coloration with p-dimethylaminobenzaldehyde with its maximum absorption at 560 nm. In the analysis of a mixture containing an amino sugar, monosaccharides, and amino acids, a colored solution with the maximum absorption at 540 nm was obtained. Thus, the results of the quantitative determination of amino sugars in such a mixture are unsatisfactory (Fig. 1).

We have proposed a modification of the Elson-Morgan method based on the differences in the extraction capacity of organic solvents in relation to the various chromophores. For this purpose, water-immiscible solvents were used. It was found that the extraction by organic solvents of the chromophores in an acid medium does not lead to the desired results. At pH > 7, the chromophore formed by amino sugars passes into such a state that is readily extracted by the organic phase, while the chromophore formed from a mixture of monosaccharide and an amino acid remains in the aqueous solution. Selective extraction with carbon tetrachloride, chloroform, and diethyl ether must be mentioned. In all cases, the degree of extraction amounts to 95-97%. On extraction of chromophore under investigation with carbon tetrachloride and with chloroform, in both cases the formation of an emulsion in the organic hase that must be centrifuged is observed. Extraction with diethyl ether leads to a clear and rapid separation of the phases, and both phases are completely

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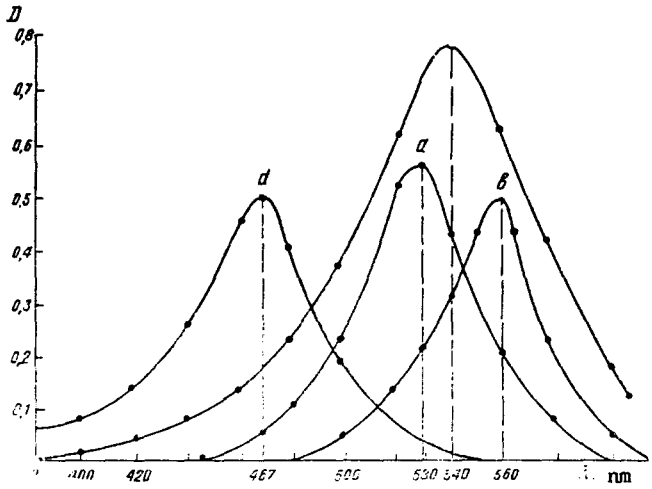

Fig. 1. Absorption spectra for amino sugars, amino acids, monosaccharides, and their mixture obtained by the Elson–Morgan method and by the method described in the present paper: a) absorption spectrum of amino sugars; b) absorption spectrum for a mixture of amino sugars + monosaccharides; c) absorption spectrum of a mixture of amino sugars, monosaccharides, and amino acids; d) absorption spectrum of the chromophore in the organic layer (λ 467 nm) obtained by the procedure described in the present paper.

TABLE 1. Quantitative Determination of Hexosamines in Standard Mixtures by Various Methods

Mixture investigated,* μg			Found by the given method, μg		
hexosamines	monosaccharides	amino acids	described	Elson–Morgan	ion-exchange [3]
5,25	15.18	200.55	5,20±0,19	7.85±0,31	5,15±0 36
10,50	20,27	93,75	10.60±0 42	17,05±0,68	10,35±0,72
20 10	25.20	85.15	20,15±0,70	37,80±0.75	19.83±1,18
60,30	30 37	70,35	60,37±1,56	150.70±3 01	58,75±3.52
80.40	40,54	45.20	80,35±1 60	160,50±3.20	78,65±3,93
100 50	60,75	20,05	100,48±2,00	130,10±2,60	98,85±4,94
150 80	100,80	15,35	150,75±3,01	180,63±3,78	147,90±7,39

*Hexosamines – glucosamine + galactosamine (1:1). Monosaccharides – glucose + mannose + frucose + galactose (1:1:1:1). Amino acids – lysine + glycine + histidine (1:1:1).

transparent, but because of the high volatility of ether all the subsequent investigations were performed with chloroform.

The spectral characteristics of the colored compound in the organic layer were determined (Fig. 1). The absorption maximum is located at 467 nm. The color is stable for four hours.

The use of the extraction-photometric method permitted the sensitivity of the analysis to be increased 10-fold.

The method has been used for the analysis of individual substances and standard mixtures containing definite amounts of amino sugars with different amounts of amino acids and monosaccharides (Table 1) and also for the analysis of various materials of bacterial origin (Table 2).

The analysis of authentic mixtures in various ratios showed that the presence of overwhelming amounts of monosaccharides and amino acids in the solution under investigation had

TABLE 2. Determination of Hexosamines in Bacterial Preparations by Various Methods

Material investigated	Found, percent				
	monosaccharides	protein	hexosamines found by given method		
			described	Elson-Morgan	ion-exch. [3]
LPS 2076-80 Vibrio alginolyticus	29,02	7.75	14,58±0,51	13,04±0,95	14,25±0 71
LPS 945-80 V. alginolyticus	35,95	7,39	5,85±0 22	15.77±0,78	5,23±0,47
PS 1385-80 V. alginolyticus	58,79	1,48	12,35±0,25	13,62±0,68	12,10±0,84
LPS 335 Yersinia enterocolitica	93,33	11,62	6,70±0,23	14,11±0.67	5 47±0 38
LPSP 1 (I peak) Y. pseudotuberculosis	38,03	31,97	8,02±0 24	10.00±0,60	7,85±0.86

*LPS – lipopolysaccharide; PS – polysaccharide; LPSP – LPS-protein complex.

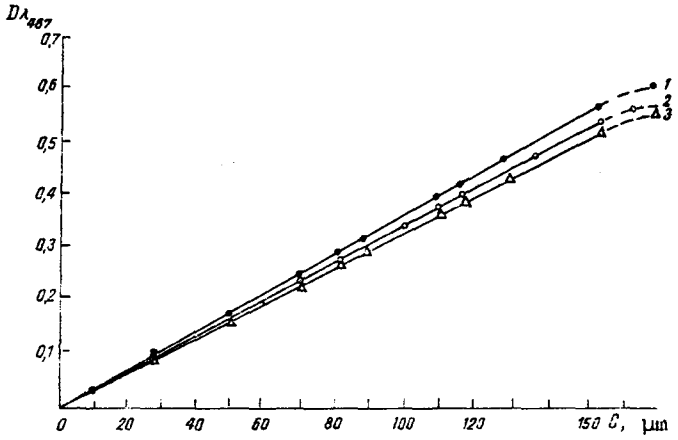

Fig. 2. Calibration curve for the determination of amino sugars: 1) D-glucosamine; 2) D-galactosamine; 3) mixture of D-glucosamine + D-galactosamine (1:1).

no influence on the results of the determination of amino sugars by our proposed modification, while the Elson–Morgan method proved to be unsatisfactory for the analysis of such mixtures (Table 1). The presence of protein in the mixture under investigation when monosaccharides were present simultaneously also considerably distorts the result of the Elson-Morgan method, while the proposed procedure eliminates this influence (Table 2). Reliable results are also given by the ion-exchange method [3], but it is extremely laborious.

The procedure described provides the possibility of determining low concentrations of amino sugars in the simultaneous presence of overwhelming amounts of monosaccharides and protein.

It was found experimentally that an optical density of 0.006 corresponds to 1 μg of amino sugars. The molar absorption coefficient is $2 \cdot 10^4$. The standard deviation calculated in each case for 10 determinations does not exceed 4%. The minimum amount of amino sugars that can be determined in the proposed method, found by Blank's method [5] is 1 μg, which shows a fairly high sensitivity of the method.

The upper limit of sensitivity due to the increase in errors at $D > 0.600$ as a consequence of the infringement of the Bouguer–Beer–Lambert law [6] is 150 μg.

Thus, we have proposed a new modification of the Elson-Morgan method which permits the quantitative determination of amino sugars in natural biopolymers in the simultaneous presence of predominating amounts of other monosaccharides and amino acids.

EXPERIMENTAL

The investigation was performed on bacterial preparations obtained in the Pacific Ocean Institute of Bioorganic Chemistry and provided by R. P. Gorshkova and T. F. Solov'eva. The preparations were used without additional purification. Before analysis, they were dried to constant weight in vacuum over phosphorus pentoxide at 50°C.

Standard samples of D-galactosamine and D-glucosamine (Chemapol, Czechoslovakia) were first crystallized until their melting points and specific rotations corresponded with those given in the literature. Standard samples of amino acids were used without additional purification. Monosaccharides and protein were determined in accordance with [7] and [8].

Procedure. To 2 ml of the solution under investigation was added 1 ml of a 1% solution of acetylacetone in 0.5 M sodium acetate solution. The mixture so obtained was heated on the boiling water bath for 20 min. After cooling, it was treated with 1 ml of the Ehrlich reagent (a solution of 400 mg of p-dimethylaminobenzaldehyde in 15 ml of ethanol and 15 ml of concentrated hydrochloric acid) and with 1 ml of ethanol, and the resulting mixture was heated in the water bath at 60-70°C for 5-10 min. The colored solution so obtained was treated with 1 ml of chloroform and, dropwise, with a saturated solution of caustic soda (to pH 8.0). After the mixture had been shaken vigorously, the chrimson coloration changed to bright yellow.

The optical density of the organic phase was measured on a VSU-2P spectrophotometer (GDR) (ℓ = 0.2 cm) at 467 nm. The amount of amino sugars in the sample being analyzed was calculated from a previously plotted calibration curve, for which standard solutions of D-galactosamine and D-glucosamine with concentrations of from 0 to 150 μg in 2 ml were used. The calibration mixtures were subjected to the procedure described above. The calibration curve is shown in Fig. 2.

Hydrolysis. The substance under investigation (1-1.5 mg) was placed in a tube, and 1 ml of 4 N hydrochloric acid was added. The tube was sealed and hydrolysis was performed at 100°C for 4 h. After the end of hydrolysis the contents of the tube were transferred quantitatively to a round-bottom flask and were evaporated to dryness in a vacuum evaporator with the addition of methanol. The dry residue was treated with 2 ml of distilled water and subjected to the analytical procedure described above.

SUMMARY

1. A modification of the method for the quantitative determination of amino sugars in polysaccharide materials of bacterial origin which excludes the influence of a mixture of amino acids and monosaccharides on the results of the analysis has been proposed.

2. The procedure that has been developed is distinguished by high specificity, sensitivity, and reproducibility of the results, and also by simplicity of performance.

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